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Research Article

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Wound healing activity of standardized brazilin rich extract from *Caesalpinia sappan* heartwood

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ABSTRACT

Wounds are common clinical entities of life which may be subacute or acute. Wound healing is a complex biochemical process where the cell structures are restored to normalcy, which depend on cell proliferation and migration, basically fibroblast cell.Plant polyphenols initiate wound healing process in natural way. The aim of this study was to evaluate the wound healing effect of standardized brazilin rich extract (BRE) from Casealpiniasappan heartwood. In vitro cell proliferation and migration of human fibroblast cell were determined by using scratch wound assay technique. Cytotoxicity, antioxidant and antibacterial activity of brazilin and BRE were also evaluated. Result indicated that brazilin and BRE had showed similar antioxidant and antibacterial activity (P > 0.05). Cytotoxicity study revealed that brazilin or BRE was nontoxic up to 500 µg/mL concentrations. Therefore, BRE can be used as potential bioactive replacer for brazilin owing to ease of production. Invitroscratch wound assay showed that BRE at 250 µg/ mL had highest cell proliferation and migration on day 2 of incubation. BRE treatment resulted in restoration of original cell number within 2 days when compared to control (P< 0.05). Hence, BRE could be used as a potential herbal compound for the treatment of wound healing. BRE could be used as a replacer for brazilin owing to its similar biological activities and ease of low cost production.

Key words: *Caesalpiniasappan* heartwood, BRE, Biological activities, Human fibroblast cell, wound healing

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INTRODUCTION

Wounds are accidental events of life, which occur due to physical, chemical and thermal injury.Wounds cause pain, bleeding, disability and often possess problems in clinical practice. A wound is a break in the normal tissue continuity, resulting in a variety of cellular and molecular sequels. Wound healing is a dynamic process involving the migration and proliferation of cells such as fibroblast, endothelial and epithelial cells, deposition of connective tissues and contraction of wound. Fibroblasts are the key cells responsible for initiating angiogenesis, epithelialization and collagen formation [1].The process of wound healing is hampered by the presence of free radicals or by microbial infection. Incomplete or prolong wound healing could create troublesome complication to health.Researches are being focused on the discovery of natural agents that promote healing and save the patient from sever complication, thereby reduce the cost of hospitalization. Herbal plants are more potent healers because they promote the repair mechanisms in the natural way [2]. Plant polyphenols are capable of promoting rapid re-epithelialisation of wounds and have antioxidant and antimicrobial properties [3].

Caesalpiniasappan L. (Leguminosae or Fabaceae) heartwood has been traditionally used as an emmenagogue, anti-inflammatory, and hemostatic agent in Southeast Asia [4]. Aqueous and ethanol extracts of CS heartwood was found to be promote the wound healing process in rats excision and incision wound model [5]. The heartwood contains various types of phenolic components, such as dibenzoxocins, flavones, homoisoflavonoids, chalcones, xanthone and brazilin [6]. Brazilin is a major active compound of *C. sappan* that has antibacterial, antioxidant and anti-inflammatory activities [7, 8, 9]. Recently, preparation and standardization of brazilin rich extract (BRE) from *C. sappan* heartwood was reported byNirmal and Panicyupakarnant [7]. We had shown that BRE has more advantage than brazilin for industrial application owing to simple and lower costs of production and could be used as an anti-acne agent.

This study was undertaken to investigate the wound healing potential of the standardized BRE. We had determined the effect of BRE on cell proliferation and migrationin human fibroblast cell (HBC) monolayer scratch model. Antioxidant, antibacterial and cytotoxicity of BRE in HBC also evaluated.

EXPERIMENTAL SECTION

Chemicals. Quercetin,2,2-diphenyl-1-picryl hydrazyl (DPPH) and sodium chloride were procured from Sigma-Aldrich (Missouri, USA). All solvents were HPLC and analytical grade and from Labscan Asia (Thailand).

Plant material.*C. sappan* heartwoods were collected from Ran SamakKee O-Sod, a Thai herbal medicine shop in Chonburi province, Thailand. A voucher specimen was identified by Dr.NileshNirmal and has been deposited at the herbarium of the Faculty of Pharmaceutical Sciences, BuraphaUniversity, Thailand. The plant material was washed and dried at 60°C for 24 h in a hot air oven, and was reduced to powder.

Isolation and identification of the brazilin. The isolation and identification of brazilin was successively carried out as per our previous report [7]. The dried powder (500 g) was successively extracted with 95% ethanol (3L × 3), under reflux conditions for 1 h, to obtain a dark brown crude extract (59.8 g) after solvent evaporation under reduced pressure. The crude extract (20 g) was fractionated by repeated silica gel column andSephadex® LH-20 columnto obtain a pure red crystal of brazilin (1140 mg).Red crystal; ^{1}H NMR (CD_3OD, 500 MHz): δ2.75 (1H, d, J = 15.8 Hz, H-7), 3.00 (1H, d , J = 15.8 Hz, H-7), 3.68 (1H, d, J = 11.1 Hz, H-6), 3.90 (1H, d, J = 11.1 Hz, H-6), 3.95 (1H, s, H-12), 6.27 (1H, d, J = 2.6 Hz, H-4), 6.45 (1H, dd, J = 8.2, 2.6 Hz, H-2), 6.58 (1H, s, H-11), 6.70 (1H, s, H-8), 7.16 (1H, d, J = 8.2, H-1).^{13}C NMR (CD_3OD, 125 MHz) δ 42.7 (C-7), 51.1 (C-12), 70.6 (C-6), 77.1 (C-6a), 103.2 (C-4), 109.2 (C-2), 112.2 (C-11), 112.6 (C-8), 115.1 (C-1a), 131.2 (C-7a), 131.2 (C-1), 137.0 (C-11a), 144.3 (C-10), 144.6 (C-9), 155.2 (C-3), 157.3 (C-4a).

Preparation of BRE.BRE was prepared and standardized to contain not less than 40% w/w brazilin. The dried CSE (50 g) was dissolved in 35% ethanol(5 L) to obtain a clear solution after filtering. The solution was loaded on a Diaion® HP-20 column (1 g CSE per 60 g resin) and eluted with 35% ethanol.The first 3 L fraction was discarded and another fraction (6 L) that contained brazilin was collected. The collected fractions were evaporated to dryness to obtain a reddish extract of BRE (24.48 g).

DPPH radical scavenging assay.DPPH radical scavenging activity was determined according to the method of Nirmal and Benjakul [10] with some modifications. Samples (1.5 mL) at concentration ranges of 1-10 µg/mL were added to 1.5 mL of 0.15 mM DPPH in 95 % ethanol. The reaction mixtures were allowed to stand for 30 min at room temperature in the dark and absorbance was measured at 517 nm using the UV-spectrophotometer. The sample blank at each concentration was prepared in the same manner except that ethanol was used instead of DPPH solution.A standard curve using quercetinin the range of 1- 10 µg/mLwas prepared and the activity was expressed as µmole QE /L.

Antibacterial assay.*Staphylococcus aureus* (NCIL 2122) and *Pseudomonas aeruginosa* (MTCC 471)were obtained from NCIM, India. Muller–Hinton agar (MHA), Muller–Hinton broth, Brain-Heart infusion (BHI) and agar were from Himedia (Mumbai, India). *S. aureus* was incubated in MHA and *P. aeruginosa* was incubated in BHA at 37 °C for 24 h. Bacterial suspension was prepared with 0.85% sodium chloride to yield approximately 10^8 CFU/ml as compared to the turbidity of the McFarland No. 0.5 standard.The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by the broth microdilution assay [11]. Ampicillin and DMSO were used as positive and negative controls, respectively.

Cell culture. Human fibroblast cell line (ATCC) was cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco ®, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco®, Grand Island, NY,USA) and antibiotics (100 U penicillin and 100 U/mL streptomycin,Gibco®, Grand Island, NY, USA) under 5% CO_2 at 37°C. The media was changed for every 2days. When the cells reached confluence, they were harvested using 0.05% trypsin–EDTA (Gibco Grand Island, NY, USA), and fresh culture medium was added for producing single cell suspensions for further incubation.

Cytotoxicity determination. Cytotoxicity of extracts was tested on human fibroblasts. Briefly different concentrations of BRE (50-500µg/mL) were prepared in PBS and were sterilized by syringe filters. Cell densities of 2 x 10^5 cells/mL were used for cytotoxic testing. BRE extracts (100µL) were added to previously seeded cells in 96 well plates of above mentioned density and incubated for 24hrs.Cell viability was tested using 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The culture was stained with MTT solution (5 mg/mL) and incubated for 4h at 37°C. After incubation, the medium was replaced with 100 µL of DMSO and absorbance was measured at 570 nm using microplate reader (Beckmen coulter).

In-vitro **wound healing assay.** Human fibroblast cells (5×10^4 cells/mL) in DMEM containing 10% FBS were seeded in a 6 well plate. After confluent cell monolayer was formed, a linear scratch was generated in the monolayer with a sterile pipette tip. Cellular debris and unattached cells were removed by washing with phosphate buffer saline (PBS) and replaced with 2 mL of DMEM containing BRE (250µg/mL) and DMEM without sample served as a control. Photographs were taken at a 10x magnification using a microphotograph (Olympus CK2, Japan) on day 0, then plates were incubated at 37°C with 5% CO_2 and photographs were taken at days 1 and 2. The images acquired for each sample were further analyzed quantitatively by using computing software (ImageJ1.42q/Java1.6.010). By comparing the images from day 0 to 2, the distance of each scratch closure 2 was determined and the percentage migration rate was calculated. In each well two scratch were made (left and right) and per scratch six points were considered. Percent migration was calculated based on previously described method.

Statistical Analyses. All analyses were performed in triplicate and a completely randomised design (CRD) was used. Analyses of variance (ANOVA) were performed and mean comparisons were done by Duncan's multiple range tests.*P* values less than 0.05 were considered statistically significant. Analysis was performed using a SPSS package (SPSS 11.0 for windows, SPSS Inc, Chicago, IL, USA).

RESULTS AND DISCUSSION

Brazilin was reported to be the major compound in *Caesalpiniasappan* heartwood. Brazilin was therefore used as an indicative marker for preparation and standardization of the active constituent rich *C. sappan* extract. Standardized brazilin rich extract (BRE) was prepared successively from crude sappan extract with not less than 40 % (w/w) content of brazilin. Nirmal and Panichayupakarant [7]reported that on the basis of yield of BRE (48 % w/w) and brazilin (6.4 % w/w), BRE has more advantage than brazilin for the cosmetics and pharmaceutical applications. In this study 49 % and 5.7 % w/w yield of BRE and brazilin was obtained from crude extract, respectively. Antioxidant activity of brazilin and BRE was evaluated in terms of DPPH radical scavenging activity (Figure 1). Increase concentrations of brazilin and BRE resulted in increase of DPPH radical scavenging activity.BRE showed slightly lower DPPH radical scavenging activity than brazilin, but not significantly difference. The structure of phenolic compounds is a key determinant of their radical scavenging which is referred as structure-activity relationships [12]. Dibenzoxocin structure of brazilin was effective for radical scavenging activity [8]. The IC_{50} values of brazilin and BRE for DPPH radical was showed to be 5.6 and 6.2 µg/mL, respectively [13]. Results indicated that BRE had comparable radical scavenging activity with that of pure brazilin and could be used as a substitute for pure brazilin.

Multiple antibiotic resistant bacteria isolated from deep and superficial human wounds were identified to be belongs to*Staphylococcus aureus* (43.7%), *Proteus mirabilis* (26.5%) and *Pseudomonas aeruginosa* (29.9%) [14]. Therefore, we determined antibacterial activity of BRE against *Staphylococcus aureus* (gram positive) and *Pseudomonas aeruginosa* (gram negative) bacteria (Table 1). Antibacterial activity shows that brazilin or BRE was most active against *S. aureus* as compared to *P. aeruginosa*. MIC or MBC value of each sample was found to be similar with each bacterium, respectively. Brazilin was found to be slightly more active than BRE against both bacterial strains. Xu and Lee [15] identified brazilin as the major antibacterial constituent of *C. sappan* heartwood.Brazilin possess strongest antibacterial activity than BRE and crude extract against acne involved bacteria including *Propionibacterium acnes*, *S. aureus* and *S. epidermidis* [7]. Antibacterial activity of phytochemicals would help for

wound healing process by avoiding infection to wound area, which could damage wound surrounding cells [14]. Therefore, brazilin and BRE can protect wound infection from wound born *S. aureus* and *P. aeruginosa* bacteria.

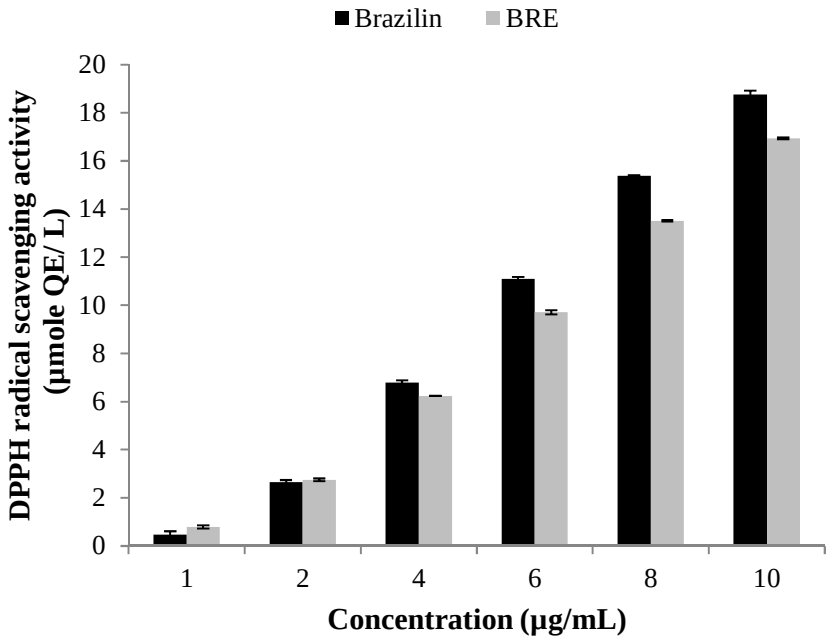

Figure 1.DPPH radical scavenging activity of brazilin and BRE. Values are mean ± SD (n=3)

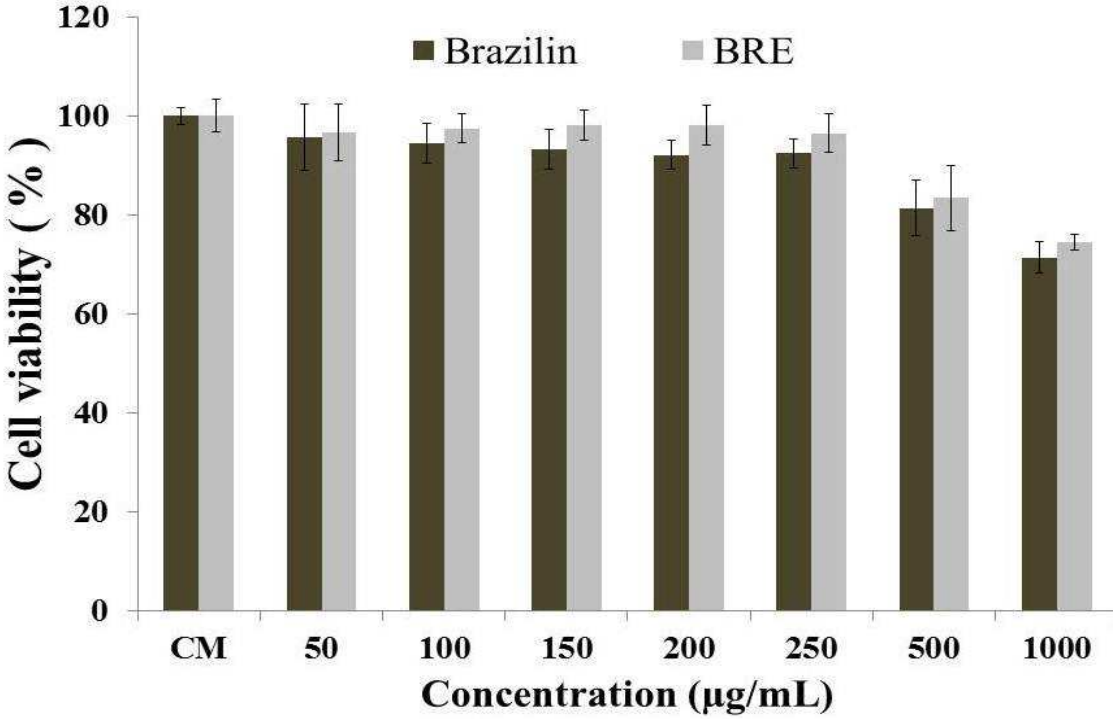

Figure 2.Cytotoxicity of brazilin and BRE on human fibroblast cell lines

Table 1: Antibacterial activities of BRE against wound involving *Staphylococcus aureus* and *Pseudomonas aeruginosa* bacteria

Microorganisms	Concentrations (µg/mL)					
	BRE		Brazilin		Ampicillin	
	MIC	MBC	MIC	MBC	MIC	MBC
S. aureus	125	125	63	63	0.1	0.1
P. aeruginosa	250	250	125	125	0.5	0.5

Cytotoxicity screening of the compounds is essential for the safety of an animal of human being. We had tested a range of brazilin or BRE concentrations (50-1000µg/mL) keeping in the mind that maximum MIC value obtained for BRE was 250µg/mL against tested bacteria. Result indicated that brazilin or BRE was non cytotoxic to the human fibroblast cell up to the 500 µg/mLconcentration (Figure 2). The percentage cell viability was above 80% indicating biocompatibility. However, brazilin or BRE exhibited cytotoxicity above500µg/mL concentration. Brazilin found to be slightly more cytotoxic as compared to BRE in the concentration range of 100-500 µg/mL ($P > 0.05$). The aqueous extract of sappan heartwood did not produce any subacute (dose 1000 mg/kg) or acute (dose 5000 mg/kg) toxicity in either sex of rats [16]. Hence brazilin or BRE could be used as safe compound for wound healing application insuring that concentration should not exceed500 µg/mL.

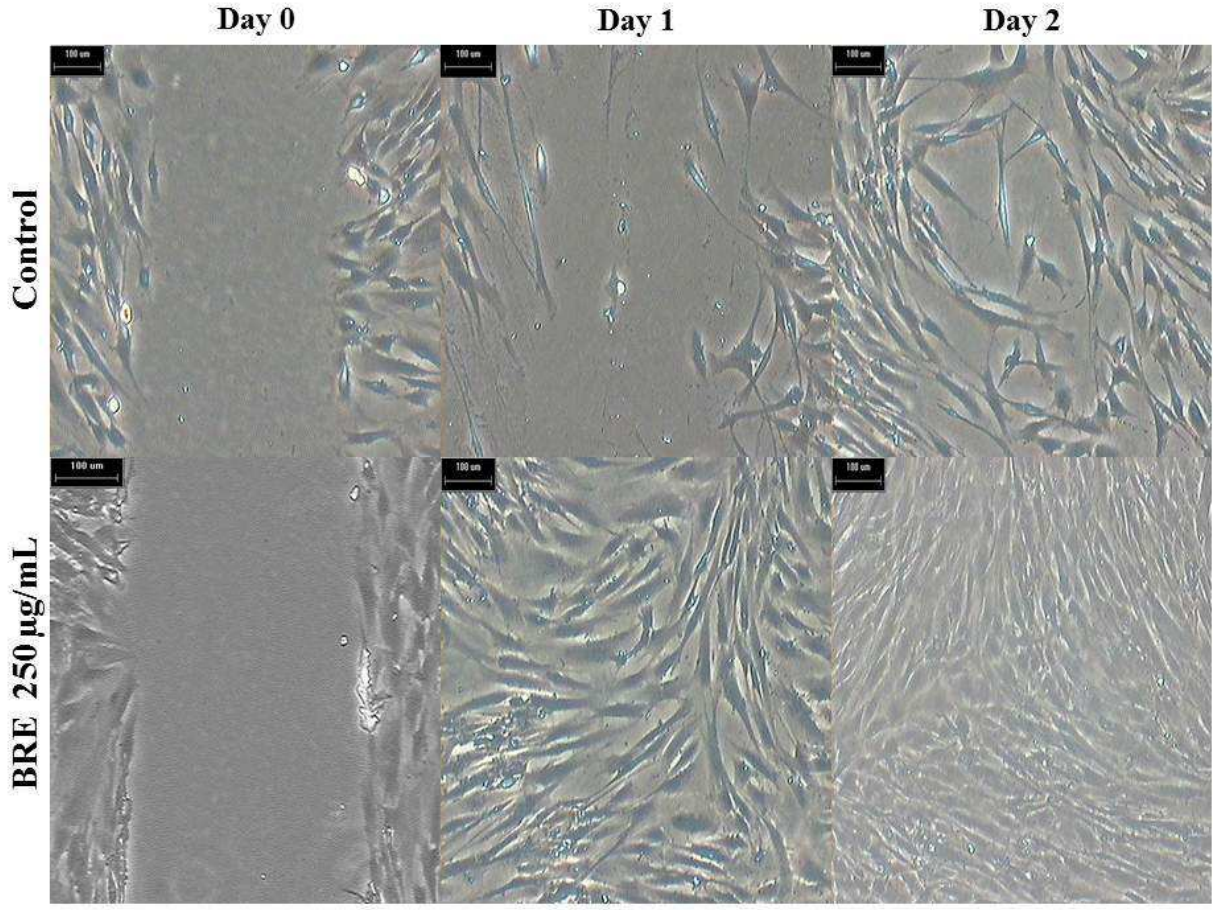

Figure 3.Cellmigration of human fibroblast cell treated with and without BRE in *invitro* scratch model.Images captured at 10× magnification using a phase-contrastmicroscope(Olympus CK2 Japan), at days 0, 1 and 2 of treatment

In-vitro scratch assay is commonly used to study cellular migration [17]. The cells on the edge of the newly created wound move toward the opening to close the "scratch" until new cellular contact is established. This event scratch closure can be visually monitored by imaging at different time points. One of the major advantages of this method is that it mimics to some extent migration of cells *in vivo*[18]. BRE was tested in order to evaluate its wound healing potential on human fibroblasts owing to simple preparation of BRE compared to brazilin. Moreover, antioxidant and

antimicrobial activity indicate that brazilin and BRE possess similar activity (P > 0.05). Cellular proliferation and migration of fibroblasts was studied in presence and absence of BRE during two days of incubation (Figure 3). The presence of BRE (250µg/mL) caused enhanced cellin the wounded area when comparing either day 1 or 2 to the control (P< 0.01). Incubation time of two days resulted in highest number of migrated cells in the wounded site treated with BRE.

The length between the scratch mark edges with BRE (250µg/mL) was 493 ± 2.1µm (day 0), 142 ± 5.2 µm (day1) and 50 ± 6.2µm (day 2), respectively(Table 2). These results were comparatively lower than that of control which indicates that presence of BRE in the culture medium enhanced the wound closure rate.

Table 2: Effect of BRE on the length of scratch closure and cell migration of human fibroblasts

Samples	Concentration (µg/ml)	Length between the scratch (µm)			Cell Migration (%)	
		Day 0	Day 1	Day 2	Day 1	Day 2
Control	Nil	418 ± 1.2b	256± 2.1a	196± 7.2a	28.6 ± 4.5y	39.4± 8.2y
BRE	250	493 ± 2.1a	142 ± 5.2b	50 ± 6.2b	80.2 ± 4.6x	87.2 ±6.7x

Different small letters within the column indicates the significant difference (P< 0.05).

Further these results can be supported by the percentage cellular migration. In presence of BRE percentage cellular migration was found to be 80.2 ±4.61 (day 1) and 87.2 ± 6.72 (day 2) when compared with control 28.6 ± 4.5 (day 1) and 39.4 ± 8.2 (day 2).BRE treatment resulted in restoration of original cell number within 2 days when compared to control. Results clearly indicate that the percentage migration was almost doubled in BRE treated group. The process of wound healing depends on cell migration and proliferation [1]. Aqueous extract of sappan heartwood (Caesalpiniaceae) (30 mg/mL) showed significant wound healing activity in rats by excision, incision and dead space wound models [5].

Oxidative stress and microbial infection have been implicated in variety of degenerative diseases including acute and chronic inflammatory conditions such as wounds [2].Phytochemicals like flavonoids, tannins and triterpenoids which are known to promote the wound healing process mainly due to their antioxidant and antibacterial property [2, 3, 14]. Brazilin has been reported to possess other bioactivities including anti-inflammatory [9], antimicrobial [15], and anti-oxidative activities [8].Based on the yield and lower cost of production, BRE has more advantage than brazilin for the industrial applications [7]. In conclusion, BRE showed comparable antioxidant and antibacterial activity with brazilin. Cytotoxic studies showed that BRE was slightly lower toxic than brazilin itself. Therefore, BRE can be more appropriate for pharmacological application such as wound healing owing to its simple and low cost of production. BRE showed significantly enhanced cell proliferation and migration of human fibroblast cell as compared to control. Hence, BRE can be used in the formulation of wound healing ointments.

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